

Andes virus M genome segment is not sufficient to confer the virulence associated with Andes virus in Syrian hamsters

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Received 26 April 2004; accepted 19 May 2004

Abstract

Sin Nombre virus (SNV) and Andes virus (ANDV), members of the genus *Hantavirus*, in the family *Bunyaviridae*, are causative agents of hantavirus pulmonary syndrome (HPS) in North and South America, respectively. Although ANDV causes a lethal HPS-like disease in hamsters, SNV, and all other HPS-associated hantaviruses that have been tested, cause asymptomatic infections of laboratory animals, including hamsters. In an effort to understand the pathogenicity of ANDV in the hamster model, we generated ANDV/SNV reassortant viruses. Plaque isolation of viruses from cell cultures infected with both parental viruses yielded only one type of stable reassortant virus: large (L) and small (S) segments of SNV and M segment of ANDV. This virus, designated SAS reassortant virus, had in vitro growth and plaque morphology characteristics similar to those of ANDV. When injected into hamsters, the SAS reassortant virus was highly infectious and elicited high-titer, ANDV-specific neutralizing antibodies; however, the virus did not cause HPS and was not lethal. These data indicate that the ANDV M genome segment is not sufficient to confer the lethal HPS phenotype associated with ANDV.

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Keywords: Andes virus; Sin Nombre virus; Hantavirus; Reassortant; Hantavirus pulmonary syndrome; Hamster

Introduction

Hantaviruses are members of the *Bunyaviridae* family of viruses. Bunyaviruses are small (80–120 nm), spherical, enveloped viruses, with genomes consisting of three single-stranded, negative-sense RNA molecules (Schmaljohn and Dalrymple, 1983). Viral RNAs are defined as small (S), medium (M), and large (L) segments that encode the nucleocapsid protein, envelope glycoproteins, and viral polymerase, respectively. Hantavirus infections are associated with two severe and sometimes fatal diseases in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (Lee et al., 1982; Nichol et al., 1993). Almost 30 different hantaviruses have been identified, over half of which are known to cause disease in humans (Nichol, 2001).

Sin Nombre virus (SNV) is the most prevalent disease-causing hantavirus in the United States. Since its appearance

in 1993, SNV has caused over 350 cases of HPS in 31 states. Despite the advanced health care that patients receive in the United States, HPS has a greater than 37% case-fatality rate (CDC, 2004). Andes virus (ANDV) is a related but distinct hantavirus that is prevalent in areas of South America (Castillo et al., 2001; Padula et al., 2000; Toro et al., 1998). SNV and ANDV share 85.5%, 77.4%, and 86.6% amino acid sequence homology in their S, M, and L open reading frames, respectively (Clustal alignment). Both viruses cause HPS with similar fatality rates in humans; however, the clinical courses of these two viral diseases differ slightly. Hemorrhagic manifestations and renal involvement have been reported more frequently in ANDV-associated HPS cases than in SNV-associated HPS cases (Castillo et al., 2001; Duchin et al., 1994). ANDV is also of significant concern to public health officials because it is the only hantavirus that has documented person-to-person transmission among family members and health-care workers (Chaparro et al., 1998; Padula et al., 1998; Toro et al., 1998).

The study of hantavirus pathogenesis and the development of medical countermeasures have been severely limited by the absence of animal models of severe HFRS or HPS.

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Report Documentation Page				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 02 JUL 2004		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Andes virus M genome segment is not sufficient to confer the virulence associated with Andes virus in Syrian hamsters, Virology 326:130 - 139				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) McElroy, AK Smith, JM Hooper, J Schmaljohn, CS				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD				8. PERFORMING ORGANIZATION REPORT NUMBER RPP-04-290	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES The original document contains color images.					
14. ABSTRACT Sin Nombre virus (SNV) and Andes virus (ANDV), members of the genus Hantavirus, in the family Bunyaviridae, are causative agents of hantavirus pulmonary syndrome (HPS) in North and South America, respectively. Although ANDV causes a lethal HPS-like disease in hamsters, SNV, and all other HPS-associated hantaviruses that have been tested, cause asymptomatic infections of laboratory animals, including hamsters. In an effort to understand the pathogenicity of ANDV in the hamster model, we generated ANDV/SNV reassortant viruses. Plaque isolation of viruses from cell cultures infected with both parental viruses yielded only one type of stable reassortant virus: large (L) and small (S) segments of SNV and M segment of ANDV. This virus, designated SAS reassortant virus, had in vitro growth and plaque morphology characteristics similar to those of ANDV. When injected into hamsters, the SAS reassortant virus was highly infectious and elicited high-titer, ANDV-specific neutralizing antibodies; however, the virus did not cause HPS and was not lethal. These data indicate that the ANDV M genome segment is not sufficient to confer the lethal HPS phenotype associated with ANDV.					
15. SUBJECT TERMS Andes virus, Sin Nombre, hantavirus pulmonary syndrome, reassortants, laboratory animals, hamsters					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 10	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

HFRS-causing hantaviruses (e.g., Hantaan virus) cause neurological disease in newborn mice (Kim and McKee, 1985; McKee et al., 1985) and recently it was reported that adult mice can develop a similar neurological disease (Wichmann et al., 2002). Signs of mild HFRS have been reported in nonhuman primates infected with Puumala virus, a virus associated with mild HFRS in humans (Groen et al., 1995; Klingstrom et al., 2002). HPS-causing hantaviruses tested, in general, do not cause disease in laboratory animals (Botten et al., 2000; Hutchinson et al., 1998, 2000). The recent discovery that Syrian hamsters develop a lethal HPS-like disease when infected with ANDV provided the first model of severe hantavirus disease for any virus in the *Hantavirus* genus (Hooper et al., 2001). In a later study, Maporal virus, a hantavirus isolated from rodents in Venezuela that is not known to cause disease in humans, also caused an HPS-like disease in Syrian hamsters (Milazzo et al., 2002).

The finding that ANDV causes lethal HPS in hamsters while a closely related hantavirus, SNV, infects but is asymptomatic (Hooper et al., 2001) led us to investigate the possibility that ANDV/SNV reassortant viruses might be used to map the virulent phenotype associated with ANDV to a particular genome segment. Evidence of naturally occurring reassortment among hantaviruses is well documented (Henderson et al., 1995; Li et al., 1995). In the laboratory, reassortants among closely related hantaviruses have been generated (Ebihara et al., 2000; Rodriguez et al., 1998). Investigators have been largely unsuccessful at generating reassortant viruses from different species of HFRS- or HPS causing hantaviruses. Kang et al. (2002) reported the generation of diploid viruses derived from HTNV and SEOV mixed infections and Rodriguez et al. (1998) described the generation of a single reassortant virus generated from cells co-infected with Black Creek Canal (BCCV) and SNV. PCR analysis indicated that the BCCV/SNV reassortant had the M segment of SNV and the L and S segments of BCCV (Rodriguez et al., 1998). In this study, we describe the generation and characterization of an ANDV/SNV reassortant. We report the use of this reassortant virus to investigate the role of the ANDV M genome segment in HPS pathogenesis in the Syrian hamster model.

Results and discussion

Co-infection of ANDV with SNV generated only one type of recoverable reassortant virus

Vero E6 cells were co-infected with ANDV and SNV at various multiplicities of infection. The progeny viruses were twice-plaque purified, amplified, and then genetically characterized by gene-specific, real-time PCR (data not shown). Only four plaques of the 208 plaques screened were found to be reassortant viruses, and all four came from the co-infections that had a ratio of 5:1 (ANDV/SNV).

The reassortant viruses that were generated were reciprocal exchanges of the M segment between the two viruses. We recovered two SAS (SNV S segment, ANDV M segment, and SNV L segment) viruses, designated SAS-23 and SAS-11; and two ASA viruses. The ASA viruses were lost on further rounds of amplification, suggesting instability of these genome segment combinations. Sequence analysis of the M gene open reading frame indicated that reassortant SAS-23 had mutations in the M segment but SAS-11 had wild-type sequence and was therefore chosen for further analysis. A parental ANDV (AAA-14) was selected for parallel analysis to ensure that the reassortant selection process did not alter the virus' characteristics in any way. No parental SNV was recovered from the dual infections. This is not surprising as our isolate of ANDV replicates more efficiently in cell culture than our isolate of SNV and we were not able to achieve high enough titers of SNV to perform a dual infection with a large excess of SNV as compared to ANDV. The parental origins of the genome segments of the reassortant viruses were confirmed by RT-PCR followed by restriction analysis (Fig. 1).

It is interesting that only M segment reciprocal exchanges occurred with significant frequency. This could suggest a co-requirement for S and L genome segments. It is thought that association of the RNA-dependent RNA polymerase (RdRp-encoded by L) with the encapsidated RNA requires sequence specificity, and recognition and displacement of the nucleocapsid protein for replication and transcription to occur (Schmaljohn and Hooper, 2001). Therefore, the recovery of M segment reassortant viruses, but not S or L segment reassortant viruses, suggests

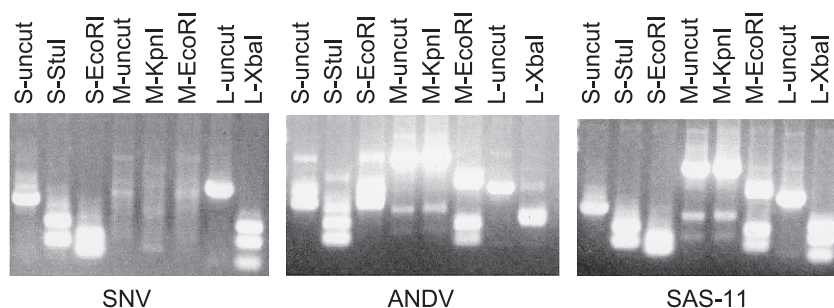


Fig. 1. Only one type of reassortant virus was obtained from co-infections of ANDV and SNV, an SAS virus. RT-PCR followed by restriction digest analysis of various viruses. The restriction patterns are shown for the parental genotypes (ANDV and SNV) and for the reassortant virus SAS-11.

that the co-requirement of S and L segments is due to the specificity of the RdRp–N interaction.

Reassortant viruses containing SNV M and ANDV S and L segments (ASA) were lost after multiple rounds of growth. One possible explanation for this instability is that the ANDV RdRp was unable to replicate or transcribe the SNV M segment with adequate efficiency due to differences in the SNV and ANDV M genome segment 5' and 3' nontranslated sequences. For example, the SNV (NM R11) and ANDV 3' vRNA nontranslated sequences differ at 11 of the 51 nucleotides (Chizhikov et al., 1995; Meissner et al., 2002). However, at this point, it is unclear if the apparent bias toward M segment monoreassortants is due to inefficient or incompatible RNA and/or protein interactions as is suggested above, or if it is simply a reflection of small sample size. Analysis of more reassortant viruses will be required to clarify this point. Small sample size could also account for that fact that, unlike others (Rodriguez et al., 1998), we did not isolate any viruses containing diploid genome segments.

SAS viruses have ANDV-like plaque morphologies

Hantaviruses do not cause lytic infections in Vero E6 cells; however, infected cells show decreased uptake of neutral red, allowing for visualization of plaques. Comparing the plaque morphology of the reassortant and parent viruses revealed that the SAS reassortant virus had large, sharply defined plaques, similar to those observed with ANDV (Fig. 2). In contrast, SNV plaques were small and poorly defined. Thus, the large, sharply defined plaque morphology associated with ANDV maps to the ANDV M genome segment.

SAS-11 reassortant virus exhibited ANDV-like growth patterns in cell culture

The SAS-11 reassortant virus exhibited ANDV-like growth patterns in culture although it replicated to slightly lower titers; 10^6 vs. 10^7 PFU/ml for parental ANDV (Fig. 2E). ANDV and SAS-11 showed peaks in virus production between days 4 and 6 postinfection (p.i.) followed by a steady decline in titer until the end of the experiment. SNV grew to peak titers of 10^5 PFU/ml on day 6 and 10 and slowly declined over the course of the infection. Thus, a partially enhanced rate-of-growth in cell culture was conferred to SNV with the substitution of an ANDV M segment.

RNA accumulation is segment-dependent

ANDV and SNV demonstrate different kinetics and quantities of RNA accumulation during in vitro infections. To determine if the kinetics of RNA accumulation were altered in the reassortant viruses, we performed reverse-transcription real-time PCR using RNA isolated from infected cells. Hantaviruses contain three negative-sense

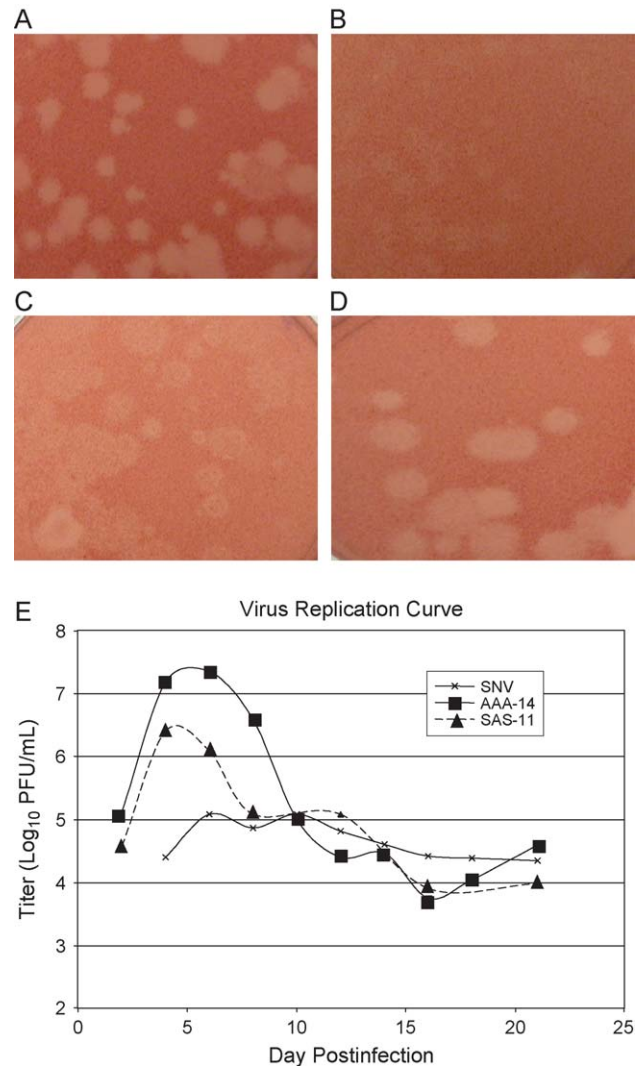


Fig. 2. The reassortant virus that contained the M segment of ANDV had plaque morphology and growth patterns similar to ANDV. (A–D) Photographs of plaque assays that were performed on the various viruses and (E) growth curves of the viruses as determined by plaque assay. (A) parental ANDV, (B) parental SNV, (C) SAS-11, and (D) AAA-14.

RNA strands; in an infected cell, both viral replication and gene expression are occurring; therefore, positive and negative sense RNAs can be detected. Consistent with the differences in titer, ANDV infections tend to produce higher levels of RNA and do so more rapidly than SNV infections (Fig. 3). The levels of S and L segment RNA for the reassortant viruses were similar to those of SNV throughout the infection (Figs. 3A,C). This is consistent with the fact that the reassortant viruses have SNV S and L segments. In contrast, the M segment RNA levels were approximately equivalent for AAA-14 and SAS-11. These data are consistent with the fact that the reassortant viruses have an ANDV M segment; however, this is a very interesting result as it suggests that the levels of a particular segment of viral RNA might be controlled in a *cis* fashion. In these experiments, the quantities and accumulation patterns of a given RNA

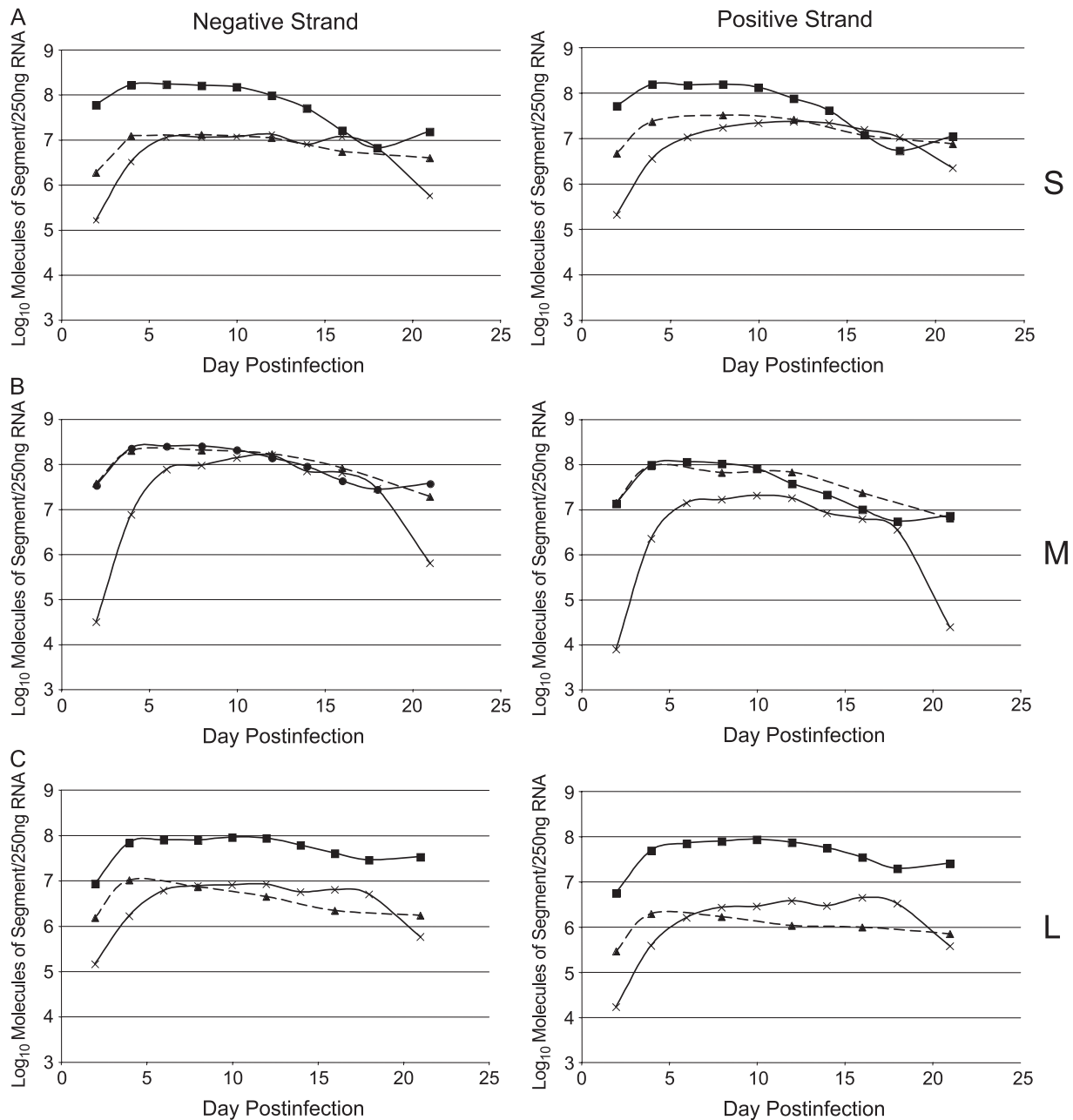


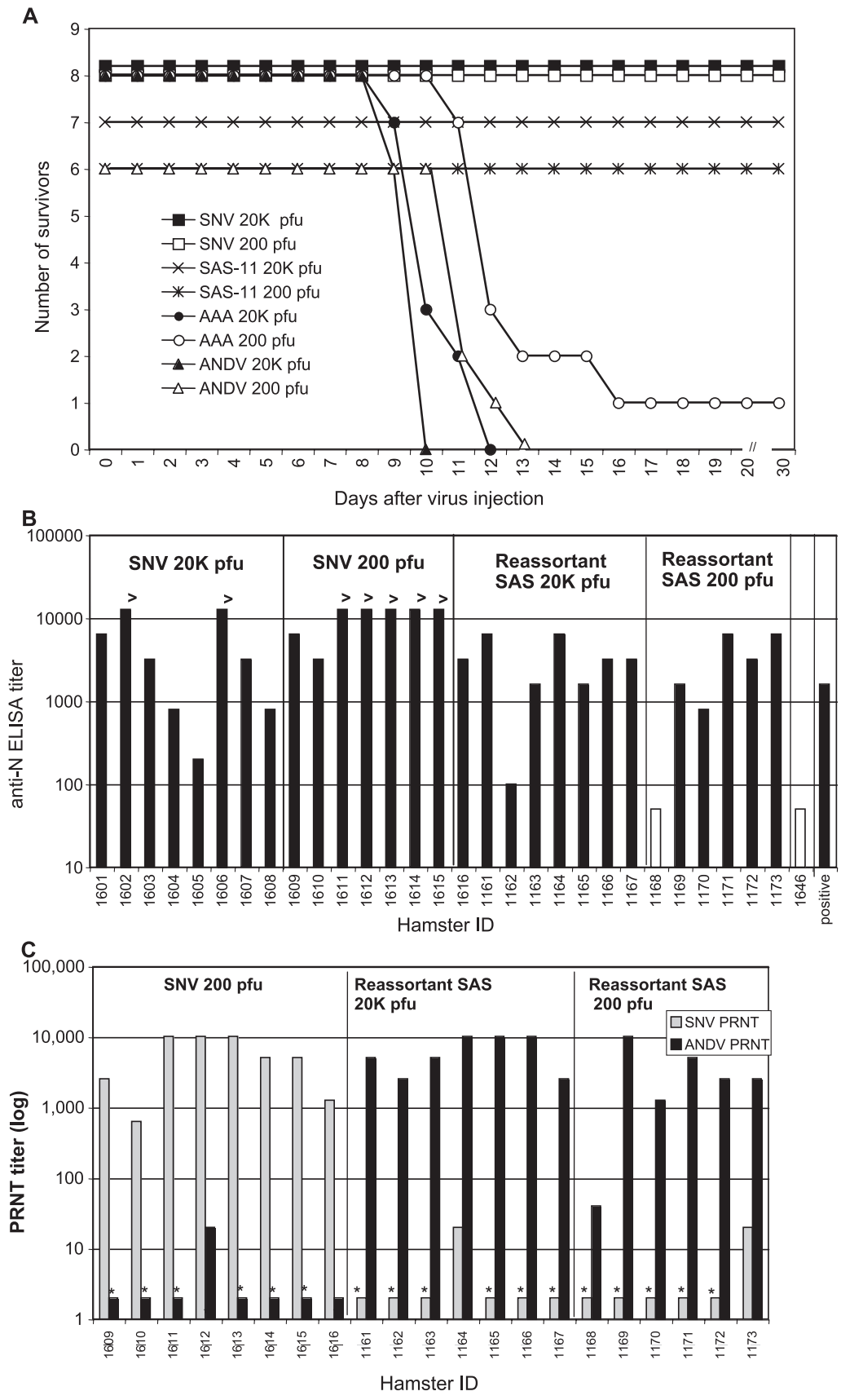
Fig. 3. Characterization of viruses by real-time PCR. The levels of the six RNA segments that are present in infected cells over a 21-day time course are shown above. (A) S Segment RNAs. (B) M segment RNAs. (C) L Segment RNAs. The negative-strand data are on the left side and the positive-strand data are on the right side. Data for each virus SAS-11 (Δ), AAA-14 (\blacksquare), and SNV (\times) are shown.

segment correlated with the genotype of the RNA segment rather than with the origin of the RdRp. As was mentioned above, this may be related to the ability of the RdRp to recognize and initiate replication or transcription from the RNA template.

SAS viruses are not lethal in Syrian hamsters

Having generated a reassortant virus containing an ANDV M genome segment in a SNV background, we were interested in exploring the possibility that the ANDV M

genome segment might be sufficient to confer the lethal HPS phenotype associated with parental ANDV. To test this possibility, we injected Syrian hamsters with a high (20,000 PFU) or low (200 PFU) dose of the reassortant or parental viruses. The SAS reassortant virus was not lethal at low or high dose (Fig. 4A). This result indicated that the ANDV M genome segment was insufficient to confer the lethal HPS phenotype. Consistent with published findings (Hooper et al., 2001), SNV did not cause illness or death at any dose, and ANDV was 100% lethal at both doses (Fig. 4A). The AAA-14 reassortant was also lethal at high and low doses



with the exception of one animal, which probably did not become infected as indicated by the absence of an antibody response after challenge (see below). The AAA-14 results demonstrate that multiple rounds of plaque purification and virus amplification did not affect the virulent phenotype associated with ANDV.

ELISA performed on sera collected from hamsters post-challenged with SAS reassortant virus had high levels of anti-nucleocapsid antibodies (Fig. 4B). Thus, the SAS reassortant virus was SNV-like in its capacity to asymptotically infect hamsters. It was possible (although highly unlikely) that the biochemistry assays used to identify the M genome segment in the SAS reassortant viruses as of ANDV origin were incorrect. We ruled this possibility out because the SAS reassortant virus elicited high-titer ANDV-specific neutralizing antibodies indicating that the M genome segment was, in fact, of ANDV origin (Fig. 4C). As a final proof, the M genome segment open reading frame in the SAS-11 reassortant virus was sequenced and found to be identical to the M genome segment of the parental ANDV (data not shown).

SAS and the pathogenesis of HPS

Although we had hoped to generate all six possible types of ANDV/SNV reassortants, after considerable effort, the only stable reassortant virus isolated was SAS. Nevertheless, this single reassortant virus has provided some interesting insights into the pathogenesis of HPS.

Tropism and virulence

For many viruses, a single protein (virulence factor) can confer a virulent phenotype on a virus by altering the tropism of the virus to a particular tissue. This was first demonstrated using reovirus reassortants whereby the neurovirulence of type 3 reoviruses in mice was mapped to the S1 genome segment, which encoded the sigma 1 attachment protein (Weiner et al., 1977). One could hypothesize that the ANDV and SNV glycoproteins might confer a different tropism in the hamster (e.g., infect different subsets of endothelial cells) and that this difference might account for the dramatic difference in pathology. However, our results indicate that the ANDV M genome segment was insufficient to account for the virulent phenotype in hamsters. Thus, although we did not rule out the possibility that the ANDV and SNV glycoproteins might confer different tropisms in the hamsters (this remains to be studied), any difference in glycoprotein-mediated tropism on its own cannot account for the virulent phenotype.

Intracellular pathology and virulence

Hantaviruses do not cause lytic infections in cell culture or in vivo; however, as can be seen in the neutral red uptake plaque assay, cells infected with hantaviruses can be functionally impaired. It is possible that HPS in hamsters is due to dysfunction of infected endothelial cells. Immunohistochemistry has demonstrated that lung endothelial cells of ANDV-infected hamsters contain viral antigen (Hooper et al., 2001). The pulmonary edema and pleural effusions associated with HPS in hamsters indicate that the endothelial cells of the lung capillaries are dysfunctional. One could hypothesize that the ANDV and SNV M genome segments confer different capacities to render an infected endothelial cell dysfunctional, and that this difference might account for the dramatic difference in pathology. Indeed, our in vitro data indicate that the ANDV M genome segment is sufficient to confer a large, sharply defined plaque morphology to the SAS reassortant viruses, and growth curves indicate that the SAS reassortant viruses grow to higher titers than SNV. However, our results in hamsters indicate that the ANDV M genome segment was insufficient to account for the virulent phenotype. Thus, although we have not ruled out the possibility that the ANDV and SNV glycoproteins might confer different capacities to render an infected endothelial cell dysfunctional, any glycoprotein-associated difference on its own cannot account for the virulent phenotype in hamsters.

Immunopathology and virulence

Many lines of evidence point toward immunopathology as the mechanism underlying HPS in humans (reviewed in Peters and Khan, 2002). One of the most compelling lines of evidence for an immune-mediated disease is the presence of activated lymphoblasts in alveoli that contain antigen in their walls and the absence of immune cells in the antigen-positive renal medulla and glomeruli (Nolte et al., 1995; Zaki et al., 1995). These pathology findings correlate with the pulmonary involvement and lack of significant renal involvement in most HPS cases. In addition, the finding that cytokine-producing cells are present in the alveolar walls and spaces of autopsy specimens has led to the hypothesis that endothelial cell dysfunction is due to the presence of cytokines that are produced by infiltrating immune cells (Mori et al., 1999). Hantavirus infection of lung microvascular endothelial cells in vitro led to the production of cytokines, but did not cause a change in the permeability of endothelial cell monolayers (Sundstrom et al., 2001) arguing against intracellular pathology caused

Fig. 4. Reassortant virus SAS was not lethal in Syrian hamsters. (A) Survival curves postchallenge (day 0) of hamsters with high and low doses of ANDV, SNV, AAA-14, and SAS-11. (B) ELISA titers against nucleocapsid protein postchallenge. Empty bars indicate titer <100 and > indicates titer ≥ 12800 . Hamster 1646, the only hamster in an ANDV group to survive, apparently was not infected. The positive control (positive) was serum collected from SNV-infected hamsters previously shown to be positive for antinucleocapsid antibodies (Hooper et al., 2001). (C) Neutralizing antibody titers. SNV and ANDV PRNT were performed on postchallenge sera from hamsters challenged with the low dose of SNV or high and low dose of SAS-11. PRNT titers represent the lowest serum dilution neutralizing 50% of the plaques in control (no serum) well. * indicates titer is <20.

by infection as the sole cause of capillary leakage. Other studies have correlated the presence of reactive oxygen/nitrogen species with HPS; however, while these studies show a strong correlation, they do not address what direct role these reactive molecules might play in altering vascular permeability (Davis et al., 2002).

The SAS reassortant virus was highly infectious and elicited a robust antiglycoprotein immune response as measured by neutralizing antibodies, and yet it did not cause HPS in hamsters. These findings demonstrated that, if immunopathology is a mechanism underlying lethal HPS in hamsters, then this immunopathology does not map to the M genome segment alone. Thus, if immunopathology is responsible for the virulent phenotype, then the ANDV S and/or L genome segments must play a role either directly by eliciting a pathogenic immune response themselves, or by modulating the immune responses to one or more ANDV-associated immunogens.

Summary

Additional reassortant viruses will be required to definitively map the ANDV/SNV strain difference in the lethal HPS phenotype in hamsters. It is possible that more than one, and perhaps all three, ANDV genes will be required for HPS. For example, suboptimal compatibility between the SNV polymerase and the ANDV M genome segment could affect the biological properties of the virus and alter the pathogenesis in the hamster. Studies of other viruses in the *Bunyaviridae* family set a precedent for the role of multiple genes in virulence. Rift Valley fever reassortant viruses demonstrated that mutations in any segment could lead to attenuation (Saluzzo and Smith, 1990). Additionally, neurovirulence of the California serogroup of bunyaviruses was independently mapped to both the M and L segments (Endres et al., 1991; Griot et al., 1993).

Our findings that the M genome segment was insufficient to confer the lethal HPS phenotype rules out several simple hypotheses of HPS pathogenesis and has turned our attention to the ANDV L and S genome segments. Perhaps the S and/or L genome segments alter the tropism or kinetics of ANDV infection, allowing it to replicate in alternative cell types or replicate more rapidly in a common cell type, thus resulting in lethal HPS in the hamster model. Alternatively, the ANDV S and/or L genome segments might prove to be more toxic in infected endothelial cells resulting in more marked dysfunction. For example, a protein product of the ANDV S or L genome is possibly responsible for its pathogenesis, as was suggested by the studies with Rift Valley fever virus, which linked expression of a nonstructural protein (NSs) with virulence (Vialat et al., 2000). No NSs proteins have ever been identified in any hantavirus despite attempts by many investigators (Parrington and Kang, 1990; Spiropoulou et al., 1994; Stohwasser et al., 1990). However, the ANDV S segment contains an ambisense ORF that is predicted to encode an approximately 10-kDa protein, and this ORF is not present in the SNV S segment. An additional mechanism

of pathogenesis might include an immune response to the ANDV nucleocapsid and/or polymerase that results in lethal immunopathology or these proteins might somehow down-regulate an otherwise protective immune response.

The Syrian hamster lethal HPS model combined with mutant viruses and reassortant viruses such as the SAS reassortant virus described here will allow us to formulate and test hypotheses and ultimately elucidate the pathogenesis of HPS in hamsters. It is our hope that this information can then be used to formulate a testable model of the pathogenesis of HPS in humans.

Materials and methods

Virus and cells

ANDV strain Chile-9717869 (obtained from T. Ksiazek, Centers for Disease Control) and SNV strain CC107 (Schmaljohn et al., 1995) were propagated in Vero E6 cells (Vero C1008 ATCC CRL 1586). Cells were maintained in Eagle minimal essential medium with Earle's salts (EMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES (pH 7.4), 200 U/ml penicillin, 200 µg/ml streptomycin, 1× nonessential amino acids (NEAA), 1.5 µg/ml amphotericin B, and 50 µg/ml gentamicin sulfate (cEMEM) at 37 °C in a 5% CO₂ incubator.

Plaque assay and isolation

Virus isolation and determination of viral titer were achieved by performing plaque assays as previously described (Schmaljohn et al., 1983). Briefly, samples were serially diluted in cEMEM, and 200 µl of each dilution was applied to duplicate wells of six-well plates containing 7-day-old Vero E6 cells. The plates were incubated for 1 h at 37 °C, with periodic rocking. Cells were then overlaid with 2 ml of 0.6% agarose in EMEM supplemented with 10% FBS, 100 U/ml of penicillin, 100 U/ml of streptomycin, 1× NEAA, and 1.5 µg/ml of amphotericin B. Cells were incubated at 37 °C for 7 days and then overlaid with the same concentration of agarose but containing 0.0165% neutral red. Plaques were counted every day for 3 days after the second overlay.

Generation of reassortant viruses

Vero E6 cells were simultaneously co-infected with twice plaque-purified stocks of ANDV and SNV at various multiplicities of infection. On days 4, 8, and 10 postinfection, supernatants were collected and cells were fed with fresh medium. Reassortant viruses were isolated by plaque assay as described above. Isolated plaques were collected by inserting a sterile 1-ml pipet tip into the plaque, and then the plaque was resuspended in 1 ml of cEMEM. The resuspended plaque was used to inoculate a T25 of Vero

E6 cells to amplify the virus. Then, the virus was subjected to a second round of plaque purification as described above.

Genetic analysis of reassortant viruses by RT-PCR and restriction digestion

RNA was isolated from infected cells on day 10 of the amplification step using TRIzol (Invitrogen). RT-PCR amplification of a portion of each segment followed by restriction digest analysis was performed. RT primers were as follows: AND/SN S RT (5'-TAG TAG TAG ACT CCT TGA GAA GC-3'), AND M RT (5'-TAG TAG TAG ACT CCG CAA G-3'), SN M RT (5'-TAG TAG TAG ACT CCG CAC G-3'), and AND/SN L RT (5'-TAG TAG TAG ACT CCG AGA TAG AGA AAG-3'). RT reactions were performed using Superscript II according to manufacturer's instructions (Invitrogen). After reverse transcription, 2 µl of each RT reaction was used as template in a PCR reaction to amplify out portions of each viral segment: S, M, and L. The other PCR reaction components were 1× HiFi buffer, 0.2 mM dNTPs, 20 ng of each primer, 2 mM MgSO₄, and 0.5 µl of platinum Taq HiFi (Invitrogen). The PCR conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and finally a 7-min extension at 72 °C. The following primers were used in the PCR reactions: AND S 135F (5'-AGT GGA GGT TGG ACC CGG ATG ACG TTA AC-3'), AND S 643R (5'-TTG TCC TAA ATC GTC CTG GCG TGA TTT C-3'), SN S 135F (5'-GGT GGA ACT GGA CCC CGA TGA TGT TAA C-3'), SN S 646R (5'-CAATTG TAC GGA ACC TCC CAG GAG TG-3'), AND M 610F (5'-GTC AGT GTT TCA ACC CAG CAC ACA-3'), AND M 1210R (5'-CTG CTA GTG TAC AAA AAA CTG TAC ATC-3'), SN M 610F (5'-GGC TAT GTT TCA TTC CAA CAC ATA C-3'), SN M 1210R (5'-CCA ATG TAC AGA AAA CTG TAC AGC C-3'), AND L 635F (5'-GTT AGA TGA GAG AGC ATC TTT GGA GGC-3'), AND L 1335R (5'-CTC CAATGT TATTTT CAA GAT TTG ATA AG-3'), SN L 636F (5'-CCT GAC GAG CGT GCA TCA TTA GAA GCC-3'), and SN L 1337R (5'-CTC ACC GAC ATT TAT TTC AAG GTT TG-3').

The S segment PCR reactions produced an approximately 500-bp fragment that, when digested by *EcoRI* or *StuI*, allowed for genetic identification. The AND S PCR product is not susceptible to digestion by *EcoRI* but a *StuI* digest resulted in two fragments: 191 and 316 bp. The SN S PCR product produced three fragments when cut by *EcoRI*: 136, 154, and 219 bp; and a *StuI* digest produced two fragments: 195 and 316 bp.

The M segment PCR reactions produced an approximately 600-bp fragment that, when digested with *EcoRI* or *KpnI*, allowed for genetic identification. The AND M PCR product was not susceptible to digestion by *KpnI* but an *EcoRI* digest produced three fragments: 137, 171, and 291 bp. The SN M PCR product was not susceptible to digestion by *EcoRI* but a *KpnI* digest produced two fragments: 239 and 361 bp.

The L segment PCR reactions produced an approximately 700-bp fragment that, when digested with *XbaI*, allowed for genetic identification. *XbaI* digestion of the AND L PCR product produced two fragments: 345 and 354 bp, while the SN L PCR product produced three fragments upon *XbaI* digestion: 347, 114, and 237 bp.

Genetic analysis of reassortant viruses by real-time PCR

RNA was isolated from infected cells using TRIzol (Invitrogen). One microgram of each RNA sample was used in an RT reaction that contained a primer specific for the negative (forward primers) or positive (reverse primers) sense strand of each of the six segments (AND or SN-S, -M, -L). The RT primers were as follows: AND S 350F (5'-CAT CTC TGA GAT ATG GGA ATG TCC TGG-3'), AND S 1114R (5'-CTT AGG TAT GAT TGG TAG AAG GCA G-3'), AND M 281F (5'-CAT ACA ACA TCT ATG GCC CAG AAG AG-3'), AND M 932R (5'-GCT GTG ATA GGT CCC ACT ATT CTT AAG-3'), AND L 5085F (5'-GCC TAC TCT TAT ATG ATG TAT TAG G-3'), AND L 5796R (5'-CCT TGT TCA TTA TGA GGG AAT ATG CAG-3'), SN S 10F (5'-ACT CCT TGA GAA GCT ACT ACG AC-3'), SN S 605R (5'-ATT GTA GAT TGA GCA GTA GGC ATG-3'), SN M 2299F (5'-GGC AGA CAG CCA AGT GTT TCT TTG-3'), SN M 3092R (5'-TCA CAC GCC TTA ATT GAA GTT ATG-3'), SN L 4119F (5'-GGA CCA ATC AAC ACC GAG TAT ATG-3'), and SN L 4892R (5'-CTC TTT AGT ACT ATA CAG CGA AAC-3'). Five microliters of each RT reaction was used in triplicate real-time PCR reactions that also contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM of gene-specific forward and reverse primers, and 200 nM of gene-specific probe. The primers and probes for each of the segments were as follows: AND S 737F (5'-GGA TGG ATC GGA TAG AAG AGT TTC-3'), AND S 803R (5'-GAG GCG ACC TTT GGC TTA GG-3'), AND S PROBE (5'-6FAM-TGG CTG CAG AGT GTC-3'), AND M 585F (5'-TTG TGT AAC AGG TCA GCT GAT TGA G-3'), AND M 658R (5'-GAG CAG GCT GAG ATA ATG TA ATG T-3'), AND M PROBE (5'-6FAM-CTG GGT TGA AAC ACT G-3'), AND L 5395F (5'-TCA ATC AAA CTC GGA GCT TAG TGT T-3'), AND L 5461R (5'-ACA TTA GGC TTA CCC CAT TCA AAA-3'), AND L PROBE (5'-6FAM-CCA GCC TCT GTT TTG A-3'), SN S 251F (5'-CAG CTC AGA AAT TGG CTT CAA A-3'), SN S 316R (5'-CCT TCA AAT GGT CAT CAG GTT CA-3'), SN S PROBE (5'-6FAM-CTG TTG ATC CAA CAG GGA-3'), SN M 2666F (5'-TGT TTG GAG ACC CTG GTG ATA TC-3'), SN M 2727R (5'-TGT GTG CTC AGG GCA TCT CA-3'), SN M PROBE (5'-6FAM-TGT CAA CAA CAA GTG GG-3'), SN L 4452F (5'-CGA TGT GAA GTA GTA CCT ACA AAA CAT G-3'), SN L 4555R (5'-ACA GCA GTA ATT GGG TTT TGA ATA GC-3'), and SN L PROBE (5'-6FAM-CAA GAA CTT TCA CAG TGC G-3'). Real-time PCR conditions were 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and

1 min at 60 °C. Reactions were run and analyzed on a 7900HT Sequence Detection System (Applied Biosystems).

Establishment of viral growth curves

Vero E6 cells were infected with parental and reassortant viruses at a moi of 0.1. Cells were collected every other day for 21 days postinfection, and supernatants were collected for plaque assay. RNA was analyzed by RT-real time PCR as described above and supernatants were analyzed for viral titer by plaque assay, also described above. RNA was isolated from the cells using TRIzol (Invitrogen) per the manufacturer's instructions.

Infection of hamsters with viruses

Adult, female, Syrian hamsters (*Mesocricetus auratus*) (Harlan) were injected intramuscularly (i.m.) (caudal thigh, 25-ga, 5/8-in. needle) with the indicated hantavirus diluted in 0.2 ml of sterile PBS (pH 7.4) as described previously (Hooper et al., 2001). A high dose and low dose were used for all of the viruses that were tested, 20,000 and 200 PFU, respectively. Work involving infected hamsters was performed in a biosafety level 4 (BSL-4) laboratory. Hamsters were monitored daily for signs of illness. Blood was collected just before challenge and on day 37 postchallenge.

ELISA and plaque reduction neutralization assay

Anti-nucleocapsid ELISA were performed as previously described (Hooper et al., 1999). The antigen was a histidine-tagged truncated Puumala virus (PUUV) nucleocapsid fusion protein expressed from pPUUSXdelta (provided by Fredrik Elgh, Umea, Sweden). Antibodies to SNV and ANDV nucleocapsid cross-react with the PUUV nucleocapsid, allowing this antigen to be used to determine relative anti-nucleocapsid responses in infected hamsters (Hooper et al., 2001). SNV and ANDV plaque reduction neutralization assays were performed as previously described (Hooper et al., 2001).

Acknowledgments

We thank Sgt. Travis Lindeblad and Elizabeth Thompson for their technical assistance. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. The research

described herein was sponsored by the Military Infectious Disease Research Program, U.S. Army Medical Research and Materiel Command, Project no. AT0002_04_RD. This work was performed while A.K.M. held a National Research Council Research Associateship Award at the U.S. Army Medical Research Institute of Infectious Diseases.

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